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Specific Chromosomal Aberrations and Amplification of the *AIB1* Nuclear Receptor Coactivator Gene in Pancreatic Carcinomas

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To screen pancreatic carcinomas for chromosomal aberrations we have applied molecular cytogenetic techniques, including fluorescent *in situ* hybridization, comparative genomic hybridization, and spectral karyotyping to a series of nine established cell lines. Comparative genomic hybridization revealed recurring chromosomal gains on chromosome arms 3q, 5p, 7p, 8q, 12p, and 20q. Chromosome losses were mapped to chromosome arms 8p, 9p, 17p, 18q, 19p, and chromosome 21. The comparison with comparative genomic hybridization data from primary pancreatic tumors indicates that a specific pattern of chromosomal copy number changes is maintained in cell culture. Metaphase chromosomes from six cell lines were analyzed by spectral karyotyping, a technique that allows one to visualize all chromosomes simultaneously in different colors. Spectral karyotyping identified multiple chromosomal rearrangements, the majority of which were unbalanced. No recurring reciprocal translocation was detected. Cytogenetic aberrations were confirmed using fluorescent *in situ* hybridization with probes for the *MDR* gene and the tumor suppressor genes *p16* and *DCC*. Copy number increases on chromosome 20q were validated with a probe specific for the nuclear receptor coactivator *AIB1* that maps to chromosome 20q12. Amplification of this gene was identified in six of nine pancreatic cancer cell lines and correlated with increased expression. *(Am J Pathol 1999, 154:525–536)*

The incidence of exocrine pancreatic cancer is about 9 in 100,000 in the USA, with a relative 5-year survival rate of less than 4%.[1] Pancreatic carcinomas contribute to 20% of cancer deaths, which is a reflection of the exceedingly poor prognosis of the disease. Cancer related symptoms occur late in disease progression. Consequently, surgical intervention remains palliative in the majority of patients.[2,3] Endoscopic retrograde pancreaticography or computed tomography assists in the diagnosis of pancreatic carcinomas. However, particularly with respect to early cancers, sensitive and specific markers are still missing. Arguably, the detection of early cancers is of high clinical interest. The identification of specific chromosomal and genetic aberrations could help to develop biomarkers for improved diagnosis and prognosis. Cytogenetic analyses of primary pancreatic carcinomas and of cell lines established from these tumors have proven to be extremely challenging because they display highly complex karyotypes and multiple marker chromosomes, which have prevented a comprehensive cytogenetic analysis of all chromosomal aberrations.[4] However, nonrandomly involved chromosomal aberrations have been identified, including the gains of chromosomes 7, 11, and 20, and losses of chromosomes 18 and 12. Chromosomal breakpoints were scattered throughout the genome.[5–7] More recently, comparative genomic hybridization (CGH) was applied to map chromosomal copy number changes in pancreatic carcinomas and cell lines established therefrom. CGH is a molecular cytogenetic screening test that allows one to survey tumor genomes for DNA gains and losses and to map regions of copy number alteration on normal metaphase chromosomes.[8] The analysis of a large number of solid tumors by CGH has invariably revealed a specific pattern of chromosomal gains and losses.[9,10] Applied to pancreatic carcinomas, several CGH studies have identified the frequent gain of chromosome arms 8q, 11q, 12p, 17q, and 20q, whereas chromosome arms 9p, 15q, and 18q were frequently lost.[11–13] CGH analyses are limited to the detection of chromosomal copy number changes, such as numerical chromosomal aberrations or intrachromosomal deletions or gene amplification. Mechanisms of chromosome copy number changes and reciprocal, balanced chromosomal aberrations remain elusive by CGH. Spectral karyotyping is a recently developed molecular cytogenetic technique that allows one to discern all chromosomes in a specific color.[14] Spectral karyotyping (SKY) is based on the simultaneous hybridization of 24 differentially labeled chromosome painting probes and spectroscopy-dependent

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color discernment. The value of SKY for the identification of hitherto unrecognized chromosomal aberrations, both numerical and structural, in cancer cells has been demonstrated.[14–18] SKY is particularly useful if numerous chromosomal aberrations, which result in highly rearranged karyotypes, impair cytogenetic evaluation. Our molecular cytogenetic analysis indicates unbalanced chromosomal aberrations, and as a result, the acquisition of copy number changes of chromosomes or chromosomal subregions are the major cytogenetic abnormalities in pancreatic carcinomas.

Materials and Methods

Cell Lines

All cell lines used in this study (BxPC-3, Capan-1, SU.86.86, Hs 766T, AsPC-1, Capan-2, MIA-PaCa-2, PANC-1, CFPAC-1) were purchased from American Type Culture Collection (Rockville, MD).

Comparative Genomic Hybridization

CGH was performed on normal, sex-matched metaphase chromosomes prepared according to standard procedures. Control DNA was labeled with digoxigenin-12-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation. Tumor DNA was extracted from pancreatic carcinoma cell lines using proteinase K digestion and phenol extraction. Labeling of genomic tumor DNA was performed by nick translation substituting dTTP by biotin-16-dUTP (Boehringer Mannheim). Differentially labeled genome (300 ng of each) was precipitated together with an excess (30 μg) of the Cot-1 fraction of human DNA (Gibco BRL, Gaithersburg, MD). The probe DNA was resuspended in 10 μl of hybridization solution (50% formamide, 2× SSC, 10% dextran sulfate), denatured (5 minutes, 75°C), and preannealed for 1 hour at 37°C. The normal metaphase chromosomes were denatured separately (70% formamide, 2× SSC) for 2 minutes at 75°C, and dehydrated through an ethanol series. Hybridization took place under a coverslip for 2 to 4 days at 37°C. Posthybridization washes and immunocytochemical detection was performed as described.[19] Biotin-labeled tumor sequences were detected with avidin conjugated to fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA), and the digoxigenin-labeled reference DNA was developed using a mouse anti-digoxin antibody, followed by a tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse antibody (Sigma, St. Louis, MO). The slides were counterstained with DAPI 4;6-diamino-2-phenylindole and embedded in an antifade solution containing para-phenylene-diamine (Sigma).

Gray level images were acquired for each fluorochrome with a cooled charge-coupled device camera (Sensys, Photometrics, Tucson, AZ) coupled to a Leica DMRXA epifluorescence microscope using sequential exposure through fluorochrome specific filters (TR1, TR2, TR3, Cy5; Chroma Technology, Brattleboro, VT) using the Leica Q-FISH ACAPS imaging system. Chromosomes were identified using DAPI-banding and co-hybridization with centromere-specific DNA probes. Fluorescence ratio images were calculated with the Cytovision CGH software (Applied Imaging, Ltd., Tyne & Wear, UK). Average ratio profiles were calculated from at least eight ratio images. An example of the results is shown in Figure 1 for the cell line AsPC-1. The vertical lines on the right side of the chromosome ideogram represent different values of the fluorescence ratios between the tumor and the normal DNA (Figure 1). The center line reflects a ratio of 1, the line left to the central line indicates the threshold for chromosome losses (ratio of 0.75), and the line on the right of the central line marks the threshold for gains (ratio of 1.25, see du Manoir et al[20] for details of the image analysis). The curves show the ratio profiles that were computed as mean values of at least 8 metaphase spreads. Average ratio profiles were the basis for the evaluation of copy number changes in all cases.

Spectral Karyotyping

Metaphase chromosomes for SKY were prepared from pancreatic cell lines according to standard procedures. SKY was performed as described.[14,21] Briefly, flow-sorted human metaphase chromosomes were amplified using degenerate oligonucleotide polymerase chain reaction (PCR).[22] Chromosome-specific painting probes were labeled by PCR with rhodamine-11-dUTP, spectrum green-dUTP, texas red-dUTP, biotin-16-dUTP, digoxigenin-11-dUTP, and combinations thereof. Two hundred ng each of the differentially labeled chromosome painting probes were precipitated in the presence of 50 μg of the Cot-1 fraction of human DNA (Gibco BRL). Hybridization took place over 2 days at 37°C. The biotinylated probe sequences were visualized using avidin Cy5 (Amersham Life Sciences, Buckinghamshire, UK), and the digoxigenin-labeled probe sequences by incubation with an anti-mouse digoxin antibody (Sigma) followed by a goat anti-mouse-antibody conjugated to Cy5.5 (Amersham Life Sciences). Chromosomes were counterstained with DAPI and mounted and coverslipped in paraphenylene-diamine (Sigma).

Image acquisition was performed using a SD200 Spectracube (Applied Spectral Imaging, Ltd., Migdal Haemek, Israel) mounted on a Leica DMRXA microscope through a custom designed optical filter (SKY-1, Chroma Technology, Brattleboro, VT). Using a Sagnac interferometer in the optical head, an interferogram was generated at all image points that is deduced from the optical path difference of the light, which in turn depends on the wavelength of the emitted fluorescence. The spectrum was recovered by Fourier transformation. The spectral information was displayed by assigning red, green, or blue colors to certain ranges of the spectrum using SkyView software (Applied Spectral Imaging). This red, green, blue-display renders chromosomes, which were labeled with spectrally overlapping fluorochromes or fluorochrome combinations, a similar color (see Figure 3B). Based on the measurement of the spectrum for each

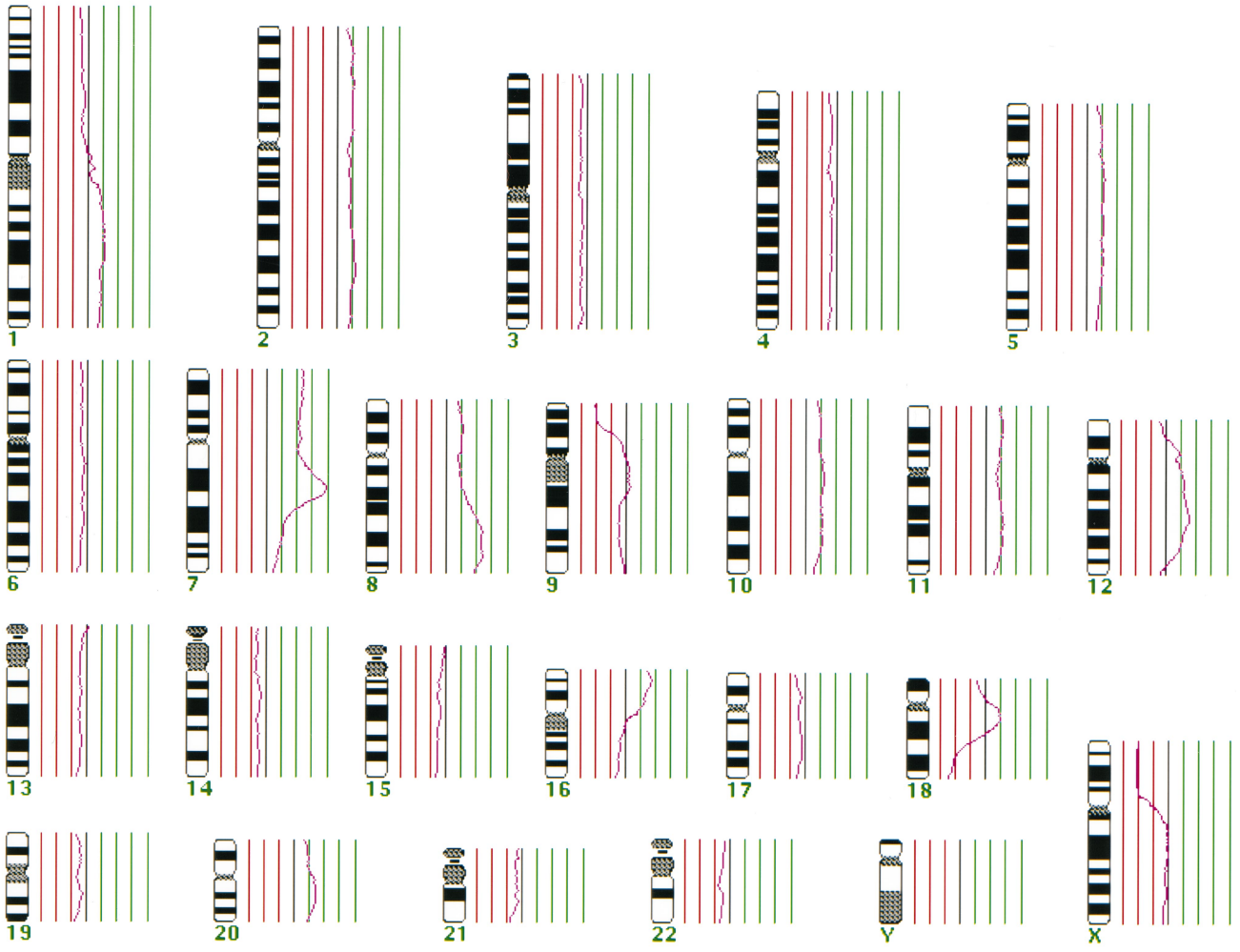

Figure 1. CGH analysis of the pancreatic carcinoma cell line AsPC-1. The red lines reflect the mean of the ratio profile calculation of eight metaphase spreads. The black center line indicates normal values, and the first red or green lines define the threshold for chromosome loss or gain. Gains of chromosomal material were mapped to 1q, 2, 5, 7, 8, 10, 11, 12q, 16p, 18q11–12, and 20. Chromosome loss occurred at 9p, 18q, and Xp.

chromosome, a spectral classification algorithm was applied to assign a pseudocolor to all points in the image that have the same spectrum.[23] This algorithm forms the basis for chromosome identification by spectral karyotyping (see Figure 3C). DAPI images were acquired from all metaphases using a DAPI-specific optical filter, inverted, and contrast enhanced using SkyView (Applied Spectral Imaging, Ltd.).

Fluorescent in Situ *Hybridization (FISH)*

Region-specific FISH probes for the following tumor suppressor genes and proto-oncogenes were performed using standard procedures. DNA-probes for the following loci were applied: *MDR* (7q21–22), *p16* (9p21), *p53* (17p13), *DCC* (18q21), *AIB1* (20q12), and a microdissection-derived painting probe for chromosome band 18q12.[24] Chromosome specific centromere probes were prepared by alphoid PCR of monochromosomal cell hybrids. Hybridization and detection followed routine FISH procedures. Images were acquired using a Leica DMRXA microscope, a cooled CCD camera (Sensys, Photometrics, Tucson, AZ), and Leica Q-FISH software. Amplification of the *AIB1* gene was scored as moderate when more than 70% of the cells revealed a ratio of higher than 2 compared with the centromere of chromosome 20 (+). Ratios that exceeded 5 were scored as high level amplifications (++). A gain of *AIB1* was defined as a simultaneous increase of *AIB1* and centromer 20 signal numbers. Signals were enumerated in 50 to 100 metaphase and interphase cells. The results were summarized in Table 1.

Northern Blot Analysis

Total RNA was isolated from cell lines using Rneasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Electrophoresis, northern transfer, and hybridization were performed by standard methods. In brief, 15 μg of RNA was size fractionated on a 1% agarose and 2.2 mol/L formaldehyde gel and transfered by alkaline method to a charged nylon membrane. The Northern blot was hybridized sequentially with a 2.2-kb fragment from the 3′ untranslated region of *AIB1* and a

Table 1. *AIB1* Amplification in Pancreatic Carcinoma Cell Lines

Cell lines	*AIB-1* amplification
BxPC-3	Gain
Capan-1	++
SU.86.86	++
Hs 766T	++
AsPC-1	Gain
Capan-2	+
MIA-PaCa-2	++
PANC-1	Gain
CFPAC-1	+

β-actin loading control probe (Clontech, Palo Alto, CA). Probes were labeled with ^{32}P by random priming.

Results

Pancreatic carcinomas are aggressive tumors that grow infiltratively in islets surrounded by normal stroma. Pure tumor cell populations are difficult to obtain, even if microdissection techniques are applied. We have therefore chosen to analyze nine cell lines established from pancreatic carcinomas. CGH was used to identify and map recurring chromosomal copy number changes. In addition, SKY was applied to six of the cell lines to delineate chromosomal aberrations in individual cells and to determine the presence of any recurring structural chromosomal aberrations. Finally, FISH with DNA probes for oncogenes and tumor suppressor genes that map to commonly altered regions in pancreatic carcinomas[25–27] was performed to confirm aberrations revealed by SKY and CGH and to map gene amplifications with higher resolution. FISH with a DNA probe for the nuclear receptor coactivator gene *AIB1*, that maps to chromosome 20q12,[28] revealed moderate to high amplification levels in six cell lines (see Figure 4 and Table 1), and Northern-blot analysis indicated increased expression of this gene (see Figure 5).

CGH

DNA from nine pancreatic carcinoma cell lines (BxPC-3, Capan-1, SU.86.86, Hs 766T, AsPC-1, Capan-2, MIA-PaCa-2, PANC-1, CFPAC-1) was analyzed using comparative genomic hybridization. All cell lines revealed copy number changes, and all chromosomes were involved in gains and losses. An example of a CGH analysis is shown in Figure 1 for the cell line AsPC-1. Chromosomal gains were identified on chromosomes and chromosomal arms and bands 1q, 2, 5, 7, 8, 10, 11, 12q, 16p, 18q11–12, and 20; chromosomal losses were mapped to 9p, 18q, and Xp. The summary of all aberrations identified in all cell lines is presented as a karyogram of chromosomal gains and losses in Figure 2. The number of chromosomal copy number changes ranged from 14 per case (AsPC-1) to 27 (Capan-2). The average number of copy alterations (ANCA) is 21.6 per case. Despite this exceedingly high number of chromosomal aberrations a clear pattern of nonrandom DNA gains and losses could be identified. Recurring gains were mapped to chromosomes and chromosome arms 5p (eight of nine cell lines), 3q (seven of nine), 7p (seven of nine), 8q (six of nine), 11q (six of nine), 20q (six of nine), 12p (five of nine), and 14q (five of nine). Consistent chromosomal losses were identified on chromosomes and chromosome arms 9p (eight of nine), 18q (eight of nine), 8p (six of nine), 17p (five of nine), 19p (five of nine), 21q (five of nine), Xp (five of nine), and Y (five of nine). The Y chromosome was lost in all cases of male patients. High-level copy number increases (amplifications) were mapped to chromosome arms and chromosome bands 5p, 5p13, 7q21–22, 8q22–24, 12p, 18q23, 19q13.1–2, 20q, and Xq24–28.

SKY

CGH identifies and maps chromosomal copy number changes; however, aberrations such as balanced chromosomal translocations cannot be detected by this technique. Conventional chromosome banding analysis can be difficult in solid tumors, in particular when highly rearranged genomes confound a complete karyotype analyses. We have therefore applied SKY to metaphase chromosomes of six of the cell lines that were also analyzed by CGH (BxPC-3, Capan-1, SU.86.86, Hs 766T, AsPC-1, MIA-PaCa-2, PANC-1). SKY allows one to simultaneously identify all human chromosomes in different colors, therefore greatly facilitating the identification of complex chromosomal aberrations. SKY is particularly useful when highly shuffled cancer chromosomes produce a banding pattern in which the origin of rearranged chromosomal material is impossible to identify with certainty. An example of the SKY analysis of a metaphase cell from cell line AsPC-1 is shown in Figure 3. In a single hybridization, all chromosomes could be identified unambiguously. Figure 3A shows a representative metaphase spread after DAPI-banding. The 24-color hybridization is displayed after visualization of the fluorescent signals by assigning a red-green-blue look-up table to certain spectral ranges (Figure 3B, see Materials and Methods). Based on the pixel by pixel spectral measurement, a pseudocolor could be assigned to all pixels that have similar spectra. This assignment is the basis for spectral karyotyping, the result of which is shown together with the banded chromosomes in Figure 3C. Based on the combination of DAPI banding and SKY, the karyotype of the diploid cell line AsPC-1 was interpreted as follows: 54,X,der(X)t(X;20)(q10;q10),+2,+5,del(7)(q22)x2,+8,der(9)t(7;9)(q31;p13)del(7)(q22),+10,+11,der(12)t(1,12,14)(q12;p12q23;q11),del(16)(q12),der(19)t(1;19)(?;q13.3),der(19)t(8;19)(q22;13.3),+20.

Table 2 presents the karyotypes for all cell lines analyzed by SKY in accordance with conventional cytogenetic nomenclature.[29]

None of the aberrations detected in the cell line AsPC-1 was a balanced translocation (Figure 3). Accordingly, the majority of the chromosomal aberrations detected by SKY is reflected by abnormal CGH profiles of the involved chromosomes. For instance, the complex

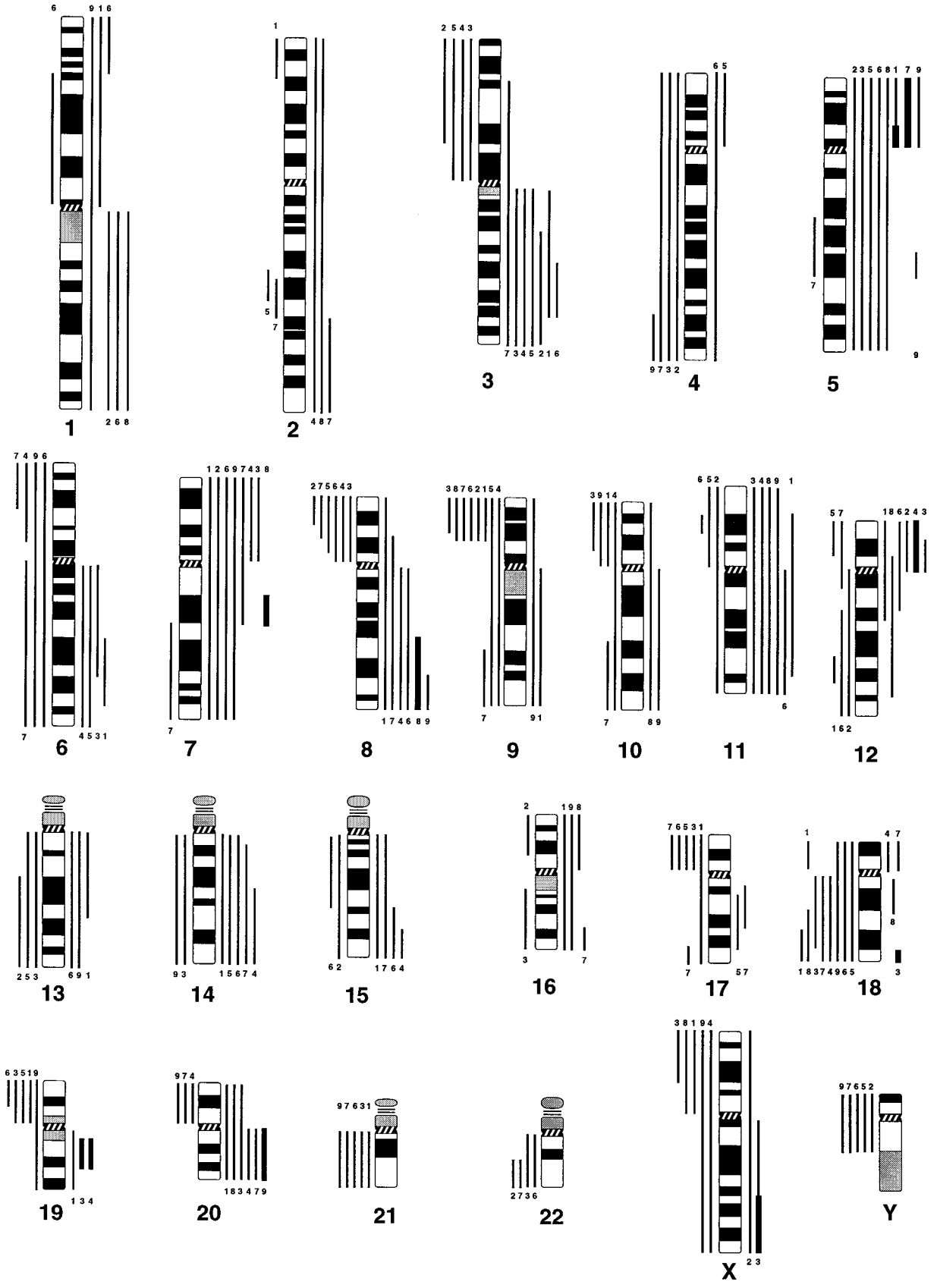

Figure 2. Karyogram of chromosomal gains and losses in nine pancreatic carcinoma cell lines. Vertical lines on the left side of each chromosome ideogram represent loss of genetic material in the tumor, whereas those on the right side correspond to a gain. Amplification sites are represented as solid squares or bars. Changes in individual cases can be identified by the case number provided on the top of each line. BxPC-3 (1), MIA-PaCa-2 (2), PANC-1(3), SU.86.86(4), CFPAC-1 (5), Capan-2 (6), Capan-1 (7), AsPC-1 (8), Hs 766T (9).

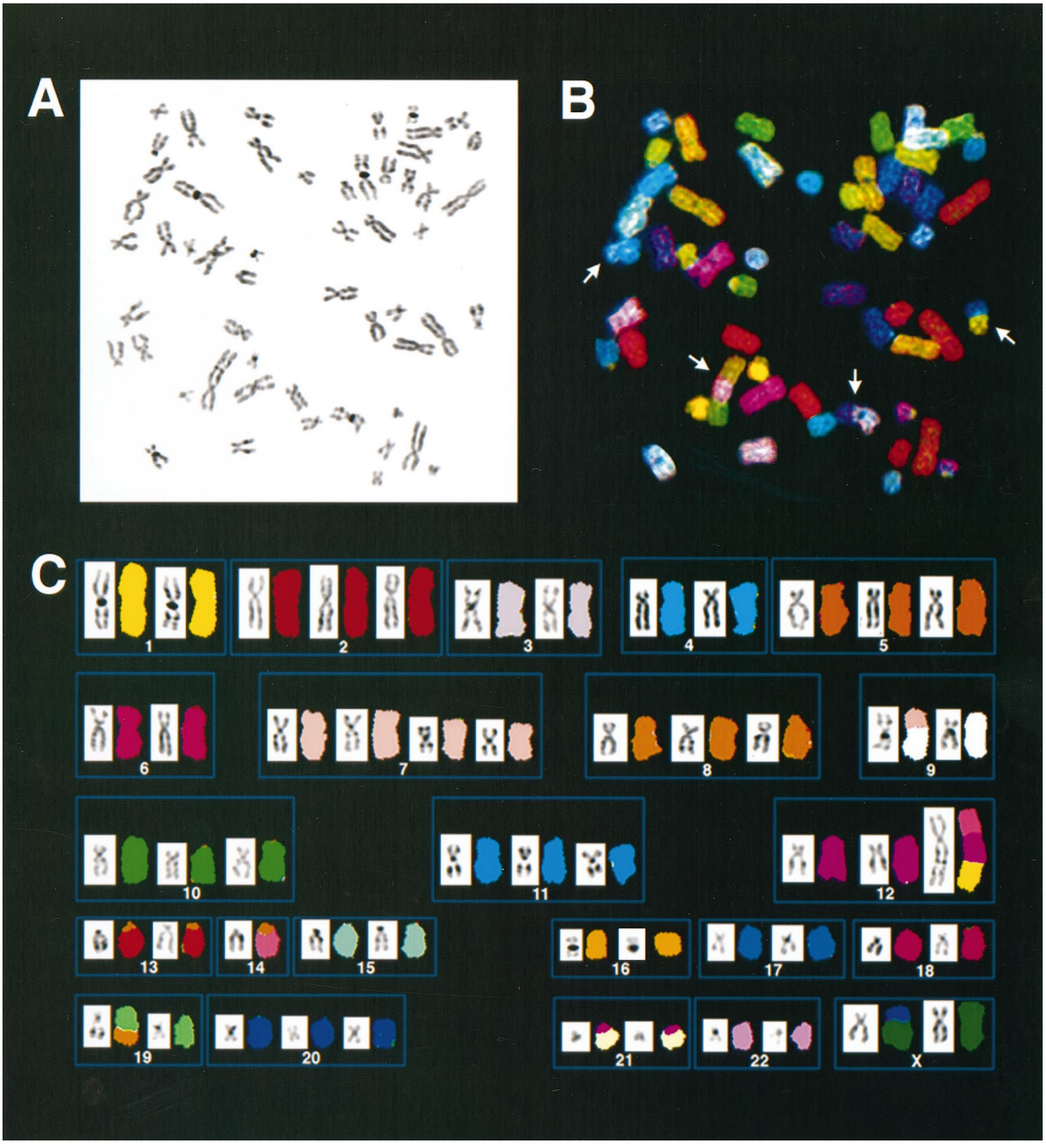

Figure 3. SKY analysis of metaphase chromosomes of AsPC-1. A: DAPI-banding of metaphase chromosomes. The image was electronically inverted. B: Same cell as in A after hybridization of 24 differentially labeled chromosome painting probes and spectral imaging. Translocation chromosomes are denoted by arrows. C: Spectral karyotype of AsPC-1. Simultaneous display of the banded and spectrally classified chromosomes. The karyotype was interpreted as: 54,X,der(X)t(X;20)(q10;q10), +2,+5,del(7)(q22)x2,+8,der(9)t(7;9)(q31;p13)del(7)(q22),+10,+11,der(12)t(1,12,14)(q12;p12q23;q11),del(16)(q12),der(19)t(1;19)(?;q13.3),der(19)t(8;19)(q22; 13.3),+20.

translocation involving chromosomes 14, 12, and 1 would predict gains on chromosomes 1q and 12; this was confirmed by CGH (Figure 1). The gain of parts of chromosome 12 coincides with translocated material from chromosome 12 that extends from 12p12 to 12q23. SKY revealed two normal copies of chromosome 7, two copies of a deleted chromosome 7 (q22), and a translocation between chromosomes 7 and 9. Again, this pattern is consistent with the CGH results that indicate an overrepresentation of the short arm of chromosome 7. The amplification on band 7q21–22, however, was only visible by CGH. FISH with a probe for the *MDR* gene gave no evidence of an amplification of this gene (not shown). Tetrasomy for part of 8q is caused by the 8;19 translocation. Loss of 9p was identified both by CGH and SKY, and so was the loss of the short arm of the X-chromosome. CGH also revealed additional information about chromosome 18; it indicated a loss of 18q21 to 18qter,

Table 2. Karyotypes of Pancreatic Carcinoma Cell Lines

Name	Karyotype based on SKY analysis
AsPC-1	54-,X, der(X)t(X;20)(q10;q10), +2,+5 +del(7)(q22)×2,+8, der(9)t(7;9)(q31;p13)del(7)(q22), +10,+11, +der(12)t(14;12;1)(q11;p12q23;q12) del(16)(q12),der(19)t(1;19)(?;q13.4), der(19)t(8;19)(q22;13.3),+20
MIA PaCa-2	62,der(X)t(X;X)(q26;p11.1), der(X)t(X;2)(p11.1;?) −Y, dic(1;16)(p10;q10), inv(2), inv(2), ins(3;X)(p13;q13q22), −4,+5 t(7;22)(q21;q12) i(8q), der(8)t(8;12)(q13;p12), dic(9;12)(q10;p12), −11, i(12p), t(14;20)(q21;q12)×2, −15,−16,−19 del(21)(q22),−22 footnote: the inv(2) have two different pericentric inversions; band assignment is not possible
PANC-1	59–62, X, del dup(X)(:p22→q28::q22→qter), −Y, t(1;5)(p22;p10) −4, der(5)t(5;19)(q11;q13), der(6)t(6;16)(6→16→6→16), +der(7)t(7;18;19)(p10;?;?), der(8)t(8;21)(q10;q10)[×2], del(9)(p11), −9, +der(11;12)(q10;p10) der(13)t(13;17;18)(p11;q?;q?), −13, −14, +del(16)(:p12→q13) −17, −18, dup(19)(q13qter), der(19)t(5;19)(?;q10) −21(3×), −22,
BxPC-3	59,X,der(X)t(X;3)(q10;q10) +der(1)t(1;7)(q10;q32), der(2)(2;12)(p23;?), der(3)t(3;6;16)(q26;?;?), +i(5p), +der(7)(7;9)(q31;q?), +8, i(9q), der(10)t(10;13), t(4;10;13)(q27;p14;q32)del(4)(q27q31), +der(11)t(10;11)(q25;q23), +del(12)(q14), +13, +14, +15, +16, +19, +20(×2)

Table 2. (continued)

Name	Karyotype based on SKY analysis
Capan-1	59–68,X,−Y, der(1)t(1;15)(q23;q15), der(1)t(1;22;10;5), del(2)(q31)×2, der(3)t(2;3)(q?;q?), inv(3)(q26q21)×2, der(3)t(3;14)(p;q), der(4)t(4;5;16)(16?::4p15→4q31::5?q), der(4)t(4:15)(q26:?), der(4;7)inv(4;7)×2, −5, inv(5)(q31q11), inv(5)(q31q11)del(q31), −6, der(6;8)t(6;8;17)(p10;q10;?), t(6;15)(q23;q21)×2, t(7;10)(q21;q24), der(8)t(8;15)(q21;?), +del(9)(p12), der(9)t(2;9)(?;q34), der(9)t(3;9)(?;q22), −10, der(10)t(5;10)(?;q24), der(11)t(11;14)(p14;?), dup(12)(q13q24)×2, −12, −13, der(14;17)t(6;17;4;17;8), der(14;17)t(8;17;14;17;8), der(14)t(7;14)(?;q21), der(14;22)t(7;14;22)(?;q14;?), der(15)t(1;15;7;5), der(17)t(15;17)(q21;p12), der(17)t(13;17)(q21;p13)×2, −20, −21
Su.86.86	67–69,XX, der(X;21)(p10;q10), der(1)t(1;(dup12;19);15)*, der(1)t(1;(dup12;19);4)†, +2, i(3q), i(6q), der(6)t(6;12)(p23;q10), der(7)t(7;15)(q31;?), der(8)t(8;22)(p10;q10), −9, −10, i(10p), der(10;18)t(q10;p10), +11, i(12p), der(12;19)t(16;(dup12;19);20)‡ i(13q), i(14q), −14, +der(16)t(10;16)(q11;q12), del(18)(q10), dup(19)(q?), i(20q), +21, i(22q)

*In three metaphases out of eleven.
†In four metaphases out of eleven.
‡In four metaphases out of eleven.

accompanied by copy number increases at chromosome bands 18q11 and 18q12. SKY and chromosome banding analyses revealed two normal chromosomes 18. We therefore used FISH with a microdissection derived painting probe for chromosome band 18q12 that explained that the apparent discrepancy was because of a duplication of chromosome band 18q12 (Figure 4, E and F). All of the aberrations detected in the cell line AsPC-1 by

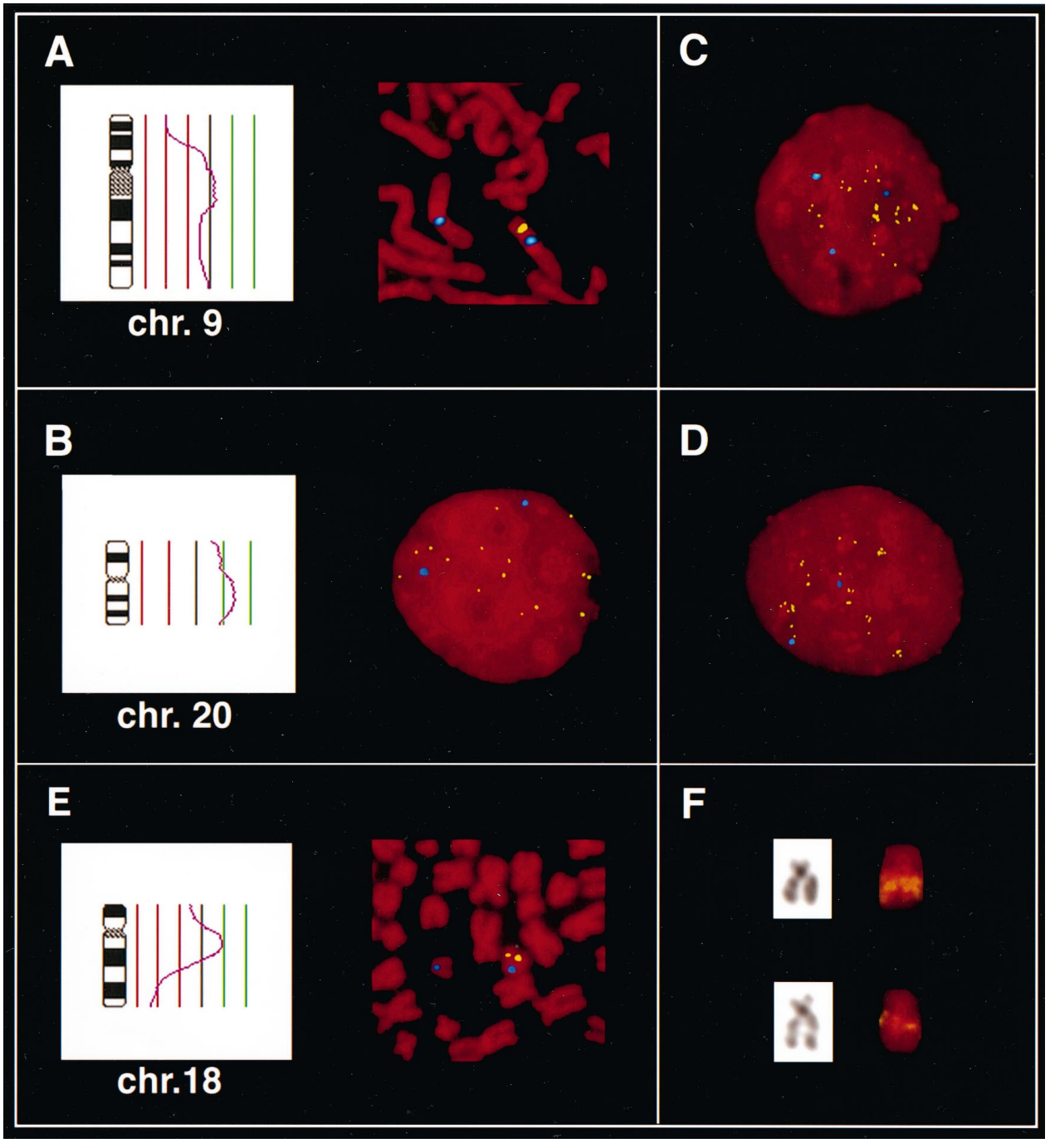

Figure 4. FISH-analyses with DNA clones for the tumor suppressor genes *DCC* and *p16* and the nuclear coactivator receptor *AIB1*. A: CGH ratio profiles from the cell line AsPC-1 for chromosome 9. The ratio profile on chromosome 9 suggests a loss of chromosome bands 9p. Dual color FISH with a centromere specific probe (blue) and a probe for *p16* (green) showed heterozygous deletion of this tumor suppressor gene. B: The ratio profile for chromosome 20 indicates a gain in the cell line MIA-PaCa-2. FISH with a probe for the *AIB1* gene indicates high-level copy number changes that are beyond the level detected by CGH. C and D: Amplification of the *AIB1* gene was also detected in the cell lines CFPAC-1 and Hs 766T. E: The ratio profile for chromosome 18 of cell line AsPC-1 indicated a gain of 18q12 accompanied by a loss of the terminal region of chromosome 18. The probe for *DCC* was deleted on one chromosome 18 in 70% of the cells of AsPC-1. F: Two apparently normal chromosome 18 were detected by both SKY and G-banding in AsPC-1. The hybridization of a microdissection-derived probe for band 18q12 clarified the cytogenetic mechanism; a duplication of this band was confirmed by the dispersed signal on the long arm of chromosome 18.

SKY were also visible by CGH, therefore all chromosomal aberrations in this cell line resulted in copy number changes. Balanced structural chromosomal aberrations are not present in the cell line AsPC-1.

In fact, comparison of the CGH and SKY data in all of the cell lines has demonstrated that chromosomal aberrations result predominantly in copy number changes: of 144 chromosomal aberrations only 6 were balanced ones. No recurring balanced chromosomal aberration was identified. Recurring breakpoints were mapped to chromosome bands 7q21 (Capan-1 and MIA-PaCA-2) and 7q31 (BxPC-3, AsPC-1, and Su.86.86).

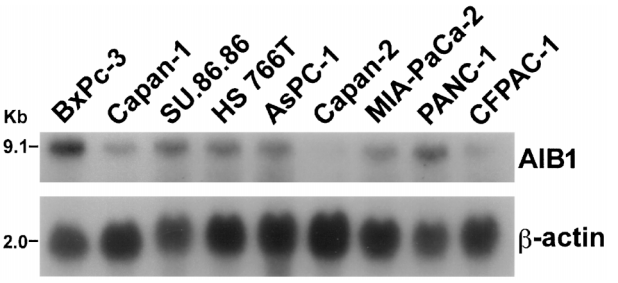

Figure 5. Northern blot analysis of RNA extracted from the nine pancreatic carcinoma cell lines shows overexpression of the AIB1 gene in seven cell lines. Capan-2 and CFPAC-1 showed expression levels that did not exceed one of the negative control (MCF10).

The cell line Su.86.86 showed a particularly interesting pattern of chromosomal aberrations. In contrast to all other cell lines analyzed, 12 of 13 translocation events involved whole arm chromosomal translocations including isochromosomes. With the exception of a translocation t(7;15)(q31;?), all translocation events were unbalanced.

FISH and Northern Blot Analysis

Based on the results obtained by CGH and SKY, we performed FISH with DNA probes for commonly involved chromosome regions on tumor metaphase chromosomes and interphase nuclei (Figure 4). For example, FISH analysis of the cell line AsPC-1 with a DNA probe for the tumor suppressor gene *p16*, which maps to chromosome band 9p21, revealed only one signal in the majority of the tumor cells (Figure 4A). The loss of the *DCC* gene, which was suggested by CGH, was confirmed as well. Thirty percent of the cell revealed homozyguous deletion of this loci (Figure 4E). This finding was confirmed by FISH analysis of islets of 10 to 20 cells of clonally derived single cells (data not shown). The potential involvement of the gene for the *MDR* gene in the amplicon on chromosome 7q21–22 could not be verified by FISH; four signals were observed in the majority of metaphase cells and interphase nuclei (data not shown).

Six of nine cell lines revealed copy number increases on chromosome arm 20q by CGH. One of the genes in this amplicon that was recently shown to be amplified in breast carcinomas is the *AIB1* nuclear receptor coactivator gene.[28] To explore the involvement of this receptor in pancreatic carcinomas we performed dual color FISH with a DNA probe for this gene and a chromosome 20 centromere probe as an internal control. Copy number increases beyond the one expected from CGH analyses were identified in the cell lines Capan-1, SU.86.86, Hs 766T, Capan-2, MIA-PaCA-2, and CFPAC-1. Examples of representative FISH experiments are displayed along with the respective CGH ratio profiles in Figure 4, B-D. The quantifications of the FISH results using a probe for the *AIB1* nuclear receptor coactivator gene are summarized in Table 1.

Based on these observations, the expression status of this recently identified gene was evaluated by Northern blot analysis. The results indicate an overexpression in seven cell lines (Figure 5), which with the exception of cell line Capan-2, correlated with the genomic amplification status.

Discussion

We have analyzed nine pancreatic carcinoma cell lines for chromosomal and genetic aberrations by CGH, SKY, and FISH with probes for candidate oncogenes and tumor suppressor genes. CGH revealed multiple chromosomal copy number changes that clustered on chromosomes 1q, 5, 7, 8, 10, 11, 12q, 16p, 18q11–12, and 20 (gains) and chromosomes 9p, 18q, and Xp (losses). The ANCA for the nine cell lines was 21.4, indicating a high degree of genetic instability. In comparison, primary colorectal carcinomas have an ANCA of 5.6,[30] small cell lung carcinomas 13,[31] and aggressive primary carcinomas of the fallopian tube an ANCA of 19.7.[32] Ten high-level copy number changes could be mapped to chromosome arms and bands 5p, 5p12–13, 7q21–22, 8q22–24, 12p, 18q23, 19q13.1–13.2, and 20q. The amplicon on chromosome 19q was also identified in previous studies of pancreatic carcinomas.[33] We have further analyzed the frequent gain and amplification of chromosome 20q using FISH and Northern blot analysis with probes for the *AIB1* nuclear receptor coactivator gene.[28] In all instances, the gain of chromosome 20q could be confirmed, however, copy number changes higher than the one expected after CGH were identified for the *AIB1* gene in cell lines Capan-1, SU.86.86, Hs 766T, MIA-PaCA-2, Capan-2, and CFPAC-1. Increased expression levels were detected in seven cell lines (Figure 5). The frequent amplification of the nuclear receptor coactivator gene *AIB1* may indicate that estrogen receptor mediated transcriptional activation confers a growth advantage even to cells that are not primarily controlled by endocrine stimuli, such as breast and ovary. Alternatively, one could hypothesize that estrogen receptors are not the only target of *AIB1*. Its interaction with CBP/p300 could also influence different signal transduction pathways,[34] which could contribute to the rapid growth kinetics of pancreatic cancers.

The frequent amplification of loci on chromosome 5p further corroborates the important involvement of genes on this chromosome arm in tumor progression. Amplifications of 5p were frequently found in advanced stage cervical cancers[35] and in lung carcinomas.[36,31] In one case, we detected an amplification that mapped to the terminal band of chromosome 18q, whereas the majority of this chromosome arm was underrepresented. The same chromosomal band was also involved in amplification events in colorectal carcinomas.[30]

The comparison of our data with data published using CGH on primary pancreatic carcinomas indicates a striking similarity of chromosomal gains and losses.[11–13] Whereas the overall number of chromosomal aberrations in our series of cell lines is higher than in primary carcinomas, the distribution of chromosomal gains and losses is virtually identical. This similarity clearly validates the use of established cell lines to identify chromosomal loci

that are recurrently involved in pancreatic carcinomas. The use of cell line DNA also omits one of the thorny problems in the analysis of these cancers. Pancreatic carcinomas show histologically a high amount of connective tissue and contaminating non-neoplastic cells. Therefore, the use of whole tissue blocks is problematic because low copy number changes may escape detection. This might explain the relatively few copy number changes that were identified by Solinas-Toldo and colleagues[11] in which 25% of the carcinomas did not show any copy number changes. Such a low number of chromosomal copy number changes would not be expected from highly aggressive and advanced pancreatic carcinomas.

The analysis of a large series of solid tumors by CGH over the past years[9,10] has led to the identification of a highly tumor specific pattern of chromosomal copy number changes. The question whether balanced chromosomal translocations are present in similar frequencies can obviously not be addressed using CGH. Conventional chromosome banding analyses of pancreatic carcinomas are difficult because the interpretation of extremely rearranged chromosomes is oftentimes impossible. However, SKY allows one to reconstruct even complex chromosomal aberrations with a high degree of accuracy because the origin of rearranged chromosomal fragments can be identified unambiguously. In an attempt to evaluate the relevance of reciprocal, balanced chromosomal aberrations in pancreatic carcinomas we have applied SKY to six of the cell lines. In general, SKY and CGH results matched closely. In the cell line AsPC-1, for instance, whole gains of chromosomes identified by CGH were because of trisomies for chromosomes 2, 5, 10, 11, and 20. Structural unbalanced chromosome translocations resulted in copy number increases, such as a marker chromosomes containing sequences of chromosome 1q and a translocation 8;19 resulted in extra copies for chromosome 8q. None of the chromosomal aberrations identified by SKY was balanced. Surprisingly, two apparently normal chromosomes 18 were identified by SKY, whereas CGH suggested the loss of the terminal bands of this chromosome along with copy number increases on band 18q11–12. We hypothesized that an explanation for this apparent discrepancy was because of a duplication of chromosome band 18q12 along with a deletion of the terminal region of the long arm. Such an aberrations would be cytogenetically indistinguishable from a normal chromosome 18. Indeed, FISH with a chromosome band-specific probe for 18q12 revealed a duplication of this region (Figure 4, E and F). The cytogenetic pattern was further clarified using a DNA probe for the *DCC* tumor suppressor gene on chromosome band 18q21 which indicated a heterozygous loss of *DCC* in 70% and homozygous loss in 30% of the cells. This finding is in agreement with previous studies regarding deletions of 18q21 in pancreatic cancers.[37] To exclude that detection artifacts contributed to the interpretation of null signal in 30% of the cells as homozygous deletions, we repeated the FISH analysis on clusters of interphase cells after seeding the cells in low density on microscopic slides. Indeed, approximately 75% of the clonally derived clusters showed one signal (with two signals for the centromer of chromosome 18), whereas in approximately one quarter of the clusters, no signal was present (data not shown).

Based on the extent of the deletion, it is likely that the *DPC4* tumor suppressor gene is lost as well.[26]

We compared the number of numerical and unbalanced structural aberrations with the number of balanced, reciprocal translocations in the six cell lines analyzed by SKY. Among the 144 chromosomal aberrations detected, only 6 were balanced, all of which were different. Only chromosome bands 7q21 and 7q31 were involved twice and three times in translocation events, respectively. Previous cytogenetic analyses suggest the involvement of these chromosome bands in translocation events.[38] Interestingly, Achille and colleagues[39] have mapped a region of frequent allelic loss in pancreatic carcinomas to this chromosomal band. This could indicate that chromosome bands that are recurrently involved in chromosomal breakpoints point to chromosomal regions that are frequently lost and, therefore, to sites of potential tumor suppressor genes. However, the sites of frequent LOH in pancreatic carcinomas, such as chromosome 9p do not coincide with chromosomal translocation events. We can therefore not conclude that the elimination of the second allele of a tumor supressor gene is caused by chromosomal translocations. However, the prevalence of unbalanced chromosomal aberrations and numerical chromosomal aberrations in pancreatic cancers indicates an aberration pattern clearly different from the one observed in hematological malignancies, in which the reciprocal exchange of chromatin seems to play a more important role.[40] Biologically, this would mean that the translocation-induced activation of oncogenes or the translocation-induced generation of a chimeric protein is less important in pancreatic tumors (and perhaps in other carcinomas as well) as compared with hematological malignancies. Dosage variations of a specific gene or a set of specific genes via copy number changes as a consequence of numerical and unbalanced structural chromosome aberrations seems to be the predominant genetic mechanism. Loss or gain of entire chromosomes, isochromosomes, insertions, deletions, and unbalanced translocations contribute to these copy number changes. In striking contrast to the variety of chromosomal aberrations observed in the pancreatic carcinomas cell lines, SKY analysis of metaphase chromosomes of Su.86.86 revealed a unique pattern of chromosomal rearrangements. The vast majority of translocation events involved whole chromosome arms, including the frequent formation of isochromosomes (Table 2). This pattern might indicate a mechanistically different generation of chromosomal aberrations in this particular cell line such as a frequent transverse division of the centromere.[41,42]

The molecular and cytogenetic analysis of tissue prepared from primary pancreatic carcinomas has proven difficult because of a strong host desmoplastic reaction and contaminating non-neoplastic cells.[43] The identification of premalignant lesions in pancreatic carcinomas remains a diagnostic challenge and the sequence of

genetic events is poorly understood compared with, eg, colorectal tumorigenesis.[44] We have previously shown that early chromosomal aberrations as determined by CGH are maintained at later stages of tumor progression.[19,30,32,45] The striking similarity of our data with data from previous studies using CGH on primary pancreatic tumors may suggest that the identification of early chromosomal aberrations in dysplastic lesions could be attempted using interphase cytogenetics with DNA probes for recurrently involved chromosomal regions directly on cytological preparations or tissue sections from bioptic material or surgically removed masses. The advantage of such an interphase approach would be that pertinent genetic markers could be directly correlated to the cellular phenotype.[46] A defined set of DNA probes for commonly involved chromosomal loci in pancreatic carcinomas could also become an important diagnostic tool which could be applied directly to fine needle aspirations and cytological preparations from endoscopic sampling.

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References

1. Riess LAG, Kosary CL, Hankey BF, Miller BA, Edwards BK: SEER Cancer Statistics Review, 1973–1995. Bethesda, MD, National Cancer Institute, Cancer Statistics Branch, 1998
2. Gudjonsson B: Carcinoma of the pancreas: critical analysis of costs, results of resections, and the need for standardized reporting. J Am Coll Surg 1995, 181:483–503
3. Conlon KC, Klimstra DS, Brennan MF: Long-term survival after curative resection for pancreatic ductal adenocarcinoma. Ann Surg 1996, 223:273–279
4. Heim S, Mitelman F: Cancer Cytogenetics: Chromosomal and Molecular Genetic Aberrations of Tumor Cells. New York, Wiley-Liss, 1995
5. Johansson B, Bardi G, Heim S, Mandahl N, Mertens F, Bak-Jensen E, Andrén-Sandberg Å, Mitelman F: Nonrandom chromosomal rearrangements in pancreatic carcinomas. Cancer 1992, 69:1674–1681
6. Bardi G, Johansson B, Pandis N, Mandahl N, Bak-Jensen E, Andrén-Sandberg Å, Mitelman F, Heim S: Karyotypic abnormalities in tumours of the pancreas. Br J Cancer 1993, 67:1106–1112
7. Griffin CA, Hruban RH, Long PP, Morsberger LA, Douna-Issa F, Yeo CJ: Chromomsome abnormalities in pancreatic adenocarcinoma. Genes Chromosomes Cancer 1994, 9:93–100
8. Kallioniemi A, Kallioniemi O-P, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 1992, 258:818–821
9. Forozan F, Karhu R, Kononen J, Kallioniemi A, Kallioniemi O-P: Genome screening by comparative genomic hybridization. Trends Genet 1997, 13:405–409
10. Ried T, Liyanage M, du Manoir S, Heselmeyer K, Auer G, Macville M, Schröck E: Tumor cytogenetics revisited: comparative genomic hybridization and spectral karyotyping. J Mol Med 1997, 75:801–814
11. Solinas-Toldo S, Wallrapp C, Müller-Pillasch F, Bentz M, Gress T, Lichter P: Mapping of chromosomal imbalances in pancreatic carcinoma by comparative genomic hybridization. Cancer Res 1996, 56: 3803–3807
12. Fukushige S, Waldman F, Kimura M, Abe T, Furukawa T, Sunamura M, Kobari M, Horii A: Frequent gain of copy number on the long arm of chromosome 20 in human pancreatic adenocarcinoma. Genes Chromosomes Cancer 1997, 19:161–169
13. Mahlamäki EH, Höglund M, Gorunova L, Karhu R, Dawiskiba S, Andrén-Sandberg Å, Kallioniemi O-P, Johansson B: Comparative genomic hybridization reveals frequent gains of 20q, 8q, 11q, 12p, and 17q, and losses of 18q, 9p, and 15q in pancreatic cancer. Genes Chromosomes Cancer 1997, 20:383–391
14. Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T: Multicolor spectral karyotyping of human chromosomes. Science 1996, 273:494–497
15. Liyanage M, Coleman A, du Manoir S, Veldman T, McCormack S, Dickson RB, Barlow C, Wynshaw-Boris A, Janz S, Wienberg J, Ferguson-Smith MA, Schröck E, Ried T: Multicolour spectral karyotyping of mouse chromosomes. Nat Genet 1996, 14:312–315
16. Veldman T, Vignon C, Schröck E, Rowley JD, Ried T: Hidden chromosomes: abnormalities in hematological malignancies detected by multicolour spectral karyotyping. Nat Genet 1997, 15:406–410
17. Ning Y, Liang JC, Fairman J, Nagarajan L, Schröck E, Ried T: Characterization of chromosome 5q deletions by subtelomeric probes and spectral karyotyping. Cancer Genet Cytogenet 1998, 103:170–172
18. Rao P, Cigudosa JC, Ning Y, Calasanz MJ, Iida S, Tagawa S, Michalei J, Klein B, Dalla-Favera R, Jhanwar SC, Ried T, Chaganti RS: Multicolor spectral karyotyping (SKY) identifies new recurring breakpoints and translocations in multiple myeloma. Blood 1998, 92:1743–1748
19. Heselmeyer K, Schröck E, du Manoir S, Blegen H, Shah K, Steinbeck R, Auer G, Ried T: Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. Proc Natl Acad Sci USA 1996, 93:479–484
20. du Manoir S, Schrock E, Bentz M, Speicher MR, Joos S, Ried T, Lichter P, Cremer T: Quantitative analysis of comparative genomic hybridization. Cytometry 1995, 19:27–41
21. Macville M, Veldman T, Padilla-Nash H, Wangsa D, O'Brien P, Schröck E, Ried T: Spectral karyotyping, a 24-colour FISH technique for the identification of chromosomal rearrangements. Histochem Cell Biol 1997, 108:299–305
22. Telenius H, Pelear AH, Tunnacliffe A, Carter NP, Behmel A, Ferguson-Smith MA, Nordenskjöld M, Pfragner R, Ponder BAJ: Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow sorted chromosomes. Genes Chromosomes Cancer 1992, 4:267–263
23. Garini Y, Macville M, du Manoir S, Buckwald RA, Lavi M, Katzir N, Wine D, Bar-Am I, Schröck E, Cabib D, Ried T: Spectral karyotyping. Bioimaging 1996, 4:65–72
24. Meltzer PS, Guan X-Y, Burgess A, Trent JM: Rapid generation of region specific probes by chromosome microdissection and their application. Nat Genet 1992, 1:24–28
25. Caldas C, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, Weinstein CL, Hruban RH, Yeo CJ, Kern SE: Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. Nat Genet 1994, 8:27–32
26. Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE: DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 1996, 271:350–353
27. Fearon ER, Cho KR, Nigro JM, Kern SE, Simon JW, Ruppert JM, Hamilton SR, Vogelstein B: Identification of a chromosome 18q21.1. Science 1990, 247:49–56
28. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Xin-Yuan G, Sauter G, Kallioniemi O-P, Trent JM, Meltzer PS: AIB1, a steroid receptor coactivator amplified in breast, and ovarian cancer. Science 1997, 277:965–968
29. Karger S: ISCN: An International System for Human Cytogenetic Nomenclature. Basel, Switzerland, 1995
30. Ried T, Knutzen R, Steinbeck R, Blegen H, Schröck E, Heselmeyer K, du Manoir S, Auer G: Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. Genes Chromosomes Cancer 1996, 15:234–245
31. Petersen I, Bujard M, Petersen S, Wolf G, Goeze A, Schwendel A, Langreck H, Gellert K, Reichel M, Just K, du Manoir S, Cremer T,

Dietel M, Ried T: Patterns of chromosomal imbalances in adenocarcinoma and squamous cell carcinoma of the lung. Cancer Res 1997, 57:2331–2335
32. Heselmeyer K, Hellström A-C, Blegen H, Schröck E, Silfverswärd C, Shah K, Auer G, Ried T: Primary carcinoma of the fallopian tube: comparative genomic hybridization reveals high genetic instability and a specific, recurring pattern of chromosomal aberrations. Int J Gynecol Pathol 1998, 17:245–254
33. Höglund M, Gorunova L, Andrén-Sandberg Å, Dawiskiba S, Mitelman F, Johansson B: Cytogenetic and fluorescence in situ hybridization analyses of chromosome 19 aberrations in pancreatic carcinomas: frequent loss of 19p13.3 and gain of 19q13.1–13.2. Genes Chromosomes Cancer 1998, 21:8–16
34. Torchia J, Rose DW, Inostroza J, Kamel Y, Westin S, Glass CK, Rosenfeld MG: The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature 1997, 387:677–684
35. Heselmeyer K, Macville M, Schröck E, Blegen H, Hellström A-C, Shah K, Auer G, Ried T: Advanced stage cervical carcinomas are defined by a recurrent pattern of chromosomal aberrations revealing high genetic instability and a consistent gain of chromosome 3q. Genes Chromosomes Cancer 1997, 19:233–240
36. Ried T, Petersen I, Holtgreve-Grez H, Speicher MR, Schröck E, du Manoir S, Cremer T: Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. Cancer Res 1994, 54:1801–1806
37. Hahn SA, Hoque ATM, Moskaluk CA, da Costa LT, Schutte M, Rozenblum E, Seymour AB, Weinstein CL, Yeo CJ, Hruban RH, Kern SE: Homozygous deletion map at 18q21.1 in pancreatic cancer. Cancer Res 1996, 56:490–494
38. Mitelman F: Catalog of chromosome aberrations in cancer. New York: Wiley-Liss, 1994
39. Achille A, Biasi MO, Zamboni G, Bogina G, Magalini AR, Pederzoli P, Perucho M, Scarpa A: Chromosome 7q allelic losses in pancreatic carcinoma. Cancer Res 1996, 56:3808–3813
40. Rowley JD: The Philadelphia chromosome translocation: a paradigm for understanding leukemia. Cancer 1990, 65:2178–2184
41. de la Chapelle A: How do isochromosomes arise? Cancer Genet Cytogenet 1982, 5:173–179
42. Horsthemke B, Gregor V, Becher R, Passarge E: Mechanism of i(6p) formation in retinoblastoma tumor cells. Cancer Genet Cytogenet 1989, 37:95–102
43. DiGiuseppe JA, Hruban RH: Pathobiology of cancer of the pancreas. Sem Surg Oncol 1995, 11:87–96
44. Wilentz RE, Hruban RH: Pathology of cancer of the pancreas. Pancreatic Cancer 1998, 7:43–65
45. Ried T, Heselmeyer-Haddad K, Schröck E, Auer G: Genomic changes defining the genesis, progression and malignancy potential in solid human tumors: a phenotype/genotype correlation. Genes Chromosomes Cancer 1999 (In press)
46. Ried T: Interphase cytogenetics and its role in molecular diagnostics of solid tumors. Am J Pathol 1998, 152:325–327